IFNγ ProSpot™ T Cell ELISpot Assay

Sample Technical Report

Study Report For:

<Company>
<Address1>
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<City> <PostCode>
<Country>

Sponsor Name:	<Sponsor Name>
Issued by:	ProImmune Limited
Study Site:	ProImmune Limited

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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

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1 QUALITY ASSURANCE

No claim of formal GLP (Good Laboratory Practice) compliance is made for this study.

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2 INTRODUCTION

Production of cytokines plays an important role in the immune response. Cytokines are involved in many different pathways including the induction of many anti-viral proteins by IFNγ, the induction of T cell proliferation by IL-2 and the inhibition of viral gene expression and replication by TNF alpha. Cytokines are not preformed factors but are rapidly produced and secreted in response to cellular activation.

ELISpot assays are widely used to monitor immune responses in both humans and animals. The method was originally developed from the standard ELISA assay by Czerkinsky *et al*.[1] as a method to enumerate antibody secretion from B cells. The assay has now been adapted to also detect secreted cytokines or antibodies from individual cells.

Essentially, the ProSpot™ T cell ELISpot assay comprises the following steps:

- A capture antibody specific for the chosen analyte is coated onto a PVDF plate
- The plate is blocked with an excess of non-specific protein, eg. serum albumin
- Cells of interest are added along with an appropriate stimulant (e.g. peptide/positive control)
- Plates are incubated and secreted cytokines are captured by the immobilized antibody on the PVDF surface
- After washing, a biotinylated detection antibody is added to allow detection of the captured cytokine
- The secreted cytokine is visualized using an avidin-HRP or avidin-ALP conjugate and a colored precipitating substrate

Each colored spot represents a cytokine-secreting cell. The spots can be counted by eye or by using an automated plate-reader. Many different cytokines can be detected using this method including IL-2, IFNγ and granzyme B. The size of the spot is an indication of the cell productivity and the avidity of the binding. The higher the avidity of the T cell recognition the higher the productivity resulting in large, well-defined spots.

The advantage of using the ProSpot™ method is that it is a more sensitive assay than other techniques such as ELISA, for detecting low frequency cytokine secreting cells, and cytokine production is correlated to individual cells. The data generated from ProSpot™ can give better quantitative and qualitative data when compared with other methods such as intracellular cytokine staining.

The tracking of immune responses following drug administration or immunization is a useful way of monitoring the effectiveness of treatments. ProSpot™ T cell ELISpot assays are an integral part of many clinical trials or basic immune monitoring research projects. They can be used in conjunction with other functional assays such as MHC multimer staining, intracellular cytokine staining and proliferation or cytotoxicity assays to gain a better overall picture of an immune response.

[1] Czerkinsky C. *et al*. (1983) A solid-phase enzyme-linked immunospot (ELISpot) assay for enumeration of specific antibody-secreting cells. J. Immunol. Methods 65: 109-21 [PubMedID: 6361139]

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2.1 PROJECT OBJECTIVE

The Sponsor requires ProImmune to carry out an interferon gamma (IFNγ) ProSpot™ T cell ELISpot assay on a single healthy donor PBMC (peripheral blood mononuclear cell) sample, in response to three controls and three viral peptides. Identical assays were carried out in three independent setups.

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3 MATERIALS

3.1 CUSTOM PEPTIDE SYNTHESIS

ProMix™ CEF peptide pool was provided by ProImmune and included in the assay as a control stimulant. The individual peptides in this pool are detailed in Table 1 below.

Table 1. Peptide Details for ProMix™ CEF Pool

Peptide	Sequence	Source	HLA Restriction
1	VSDGGPNLY	Influenza A	HLA-A1
2	CTELKLSDY	Influenza A	HLA-A1
3	GILGFVFTL	Influenza M	HLA-A2
4	FMYSDFHFI	Influenza A	HLA-A2
5	CLGGLLTMV	EBV LMP2A	HLA-A2
6	GLCTLVAML	EBV BMLF1	HLA-A2
7	NLVPMVATV	HCMV pp65	HLA-A2
8	KTGGPIYKR	Influenza NP	HLA-A68
9	RVLSFIKGTK	Influenza NP	HLA-A3
10	ILRGSVAHK	Influenza A	HLA-A3
11	RVRAYTYSK	EBV	HLA-A3
12	RLRAEAQVK	EBV	HLA-A3
13	SIIPSGPLK	Influenza M	HLA-A3/A11
14	AVFDRKSDAK	EBV EBNA 4NP	HLA-A11
15	IVTDFSVIK	EBV	HLA-A11
16	ATIGTAMYK	EBV	HLA-A11
17	DYCNVLNKEF	EBV RTA	HLA-A24
18	LPFDKTTVM	Influenza NP	HLA-B7
19	RPPIFIRRL	EBV	HLA-B7
20	ELRSRYWAI	Influenza NP	HLA-B8
21	RAKFKQLL	EBV BZLF-1	HLA-B8
22	FLRGRAYGL	EBV EBNA 3A	HLA-B8
23	QAKWRLQTL	EBV EBNA 3	HLA-B8
24	SDEEEAIVAYTL	HCMV	HLA-B18
25	SRYWAIRTR	Influenza NP	HLA-B27
26	ASCMGLIY	Influenza A	HLA-B27
27	RRIYDLIEL	EBV EBNA 3C	HLA-B27
28	YPLHEQHGM	EBV EBNA 3A	HLA-B35
29	IPSINVHHY	HCMV pp65	HLA-B35
30	EENLLDFVRF	EBV	HLA-B44
31	EFFWDANDIY	HCMV	HLA-B27
32	TPRVTGGGAM	HCMV	HLA-B7

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Test stimulants used in this study are detailed in Table 2 below; they were tested at a final concentration of 5μM.

Table 2. Details of Test Stimulants

ID	Viral Epitope	Sequence	MHC Restriction	MW (g/mol)	Purity (%)	Description
2	Flu MP 58-66	GILGFVFTL	A*02:01	966.2	90.12	Reconstituted 2.0mg of peptides 2, 4 and 6 in DMSO to give 10mM stock, which was further diluted to final working concentration in assay media.
4	EBV BMLF1 259	GLCTLVAML	A*02:01	920.2	88.58	
6	HCMV pp65 495	NLVPMVATV	A*02:01	943.18	94.44	
8	Negative	Proprietary information	A*02:01	N/A	N/A	N/A

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3.2 CELL PREPARATION

PBMC samples from healthy human donors were isolated from whole blood and cryopreserved in liquid nitrogen (vapor-phase). Cells were thawed into pre-warmed media. Details of donor cell counts and viability are listed in Table 3 below.

Table 3. Cell Counts and Viability for ProSpot™ T Cell ELISpot Assay

Donor No.	Donor ID	Total Live Cell Count (x 10^6)	% Viability	No of cells /well (x 10^3)
D01	BD02_Setup 1	50.0	81.0	178.0
D02	BD02_Setup 2	54.0	86.0	155.5
D03	BD02_Setup 3	58.0	88.0	163.0

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4 IFNγ PROSPOT™ T CELL ELISPOT ASSAY

Multi-well PVDF membrane plates were initially coated with anti-IFNγ capture antibody. PBMCs were incubated with control or test stimulants (peptides) for 18 hours; typically cells are plated at between 60,000 – 250,000 cells per well. Specific details of the cell samples and input numbers per well are shown in Table 3.

Each donor test series was carried out in sextuplet wells and included an unstimulated (media alone) control series. Control stimulants were also included in the study to demonstrate the functional capacity of donor cell samples to secrete IFNγ cytokine.

- Phytohemagglutinin (PHA) is a lectin derived from *Phaseolus vulgaris* that mimics T cell activation through the T cell receptor. It is used at a final assay concentration of 2.5µg/ml. A PHA response is normally expected in all donors.
- The CEF peptide pool consists of 32 different MHC Class I restricted peptides, all defined as common $CD8^+$ T cell epitopes derived from cytomegalovirus (CMV), Epstein-Barr virus (EBV) and influenza virus. It is used at a final assay concentration of 2.5µM. Up to 90% of donors might be expected to respond significantly to CEF.

Following incubation, cells were washed off and the assay proceeded with primary and secondary anti-IFNγ detection. Freshly prepared BCIP/NBT substrate was used to reveal spot formation. Spots were counted using an automated ELISpot plate reader.

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4.1 ANALYSIS

4.1.1 Graphical Representation of IFNγ ProSpot™ T Cell ELISpot Data

The number of spot-forming cells (SFCs) for IFNγ cytokine secretion from test wells was counted and adjusted to SFCs per million total cells. Results from individual donors are illustrated below.

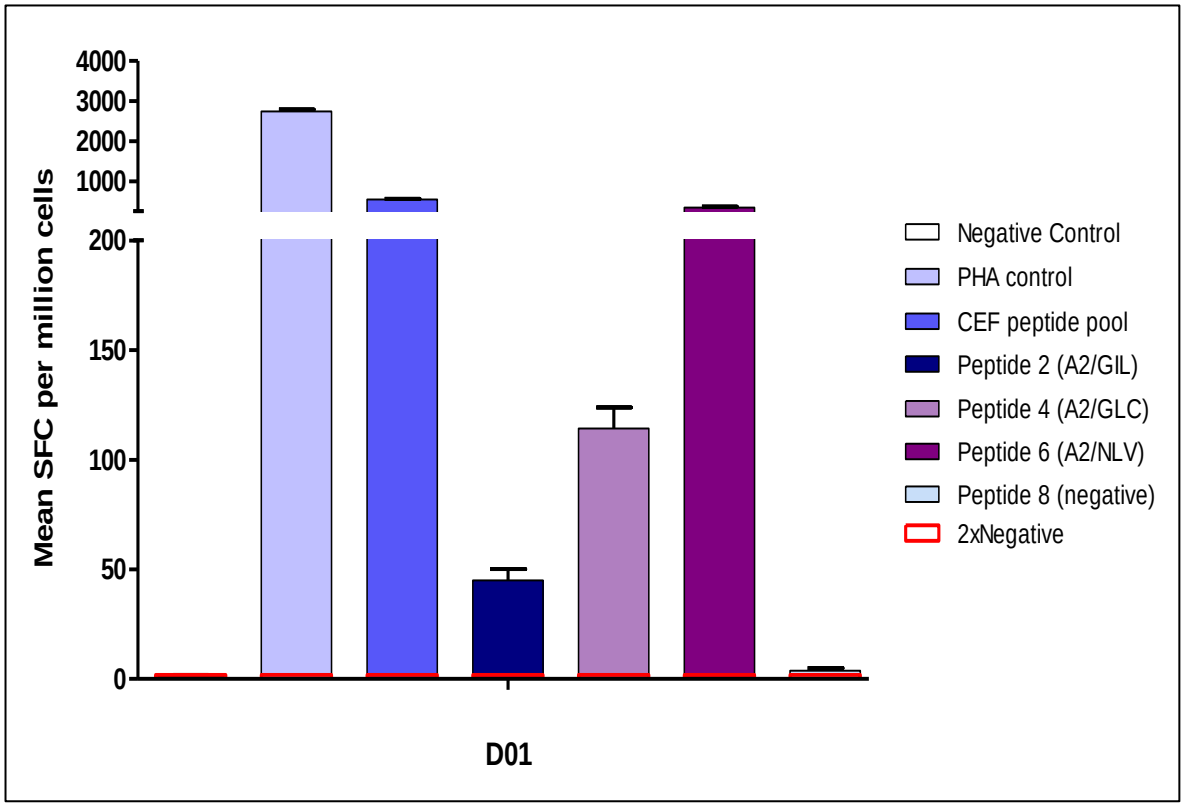

Figure 1: Number of SFC per Million Cells for Sample D01

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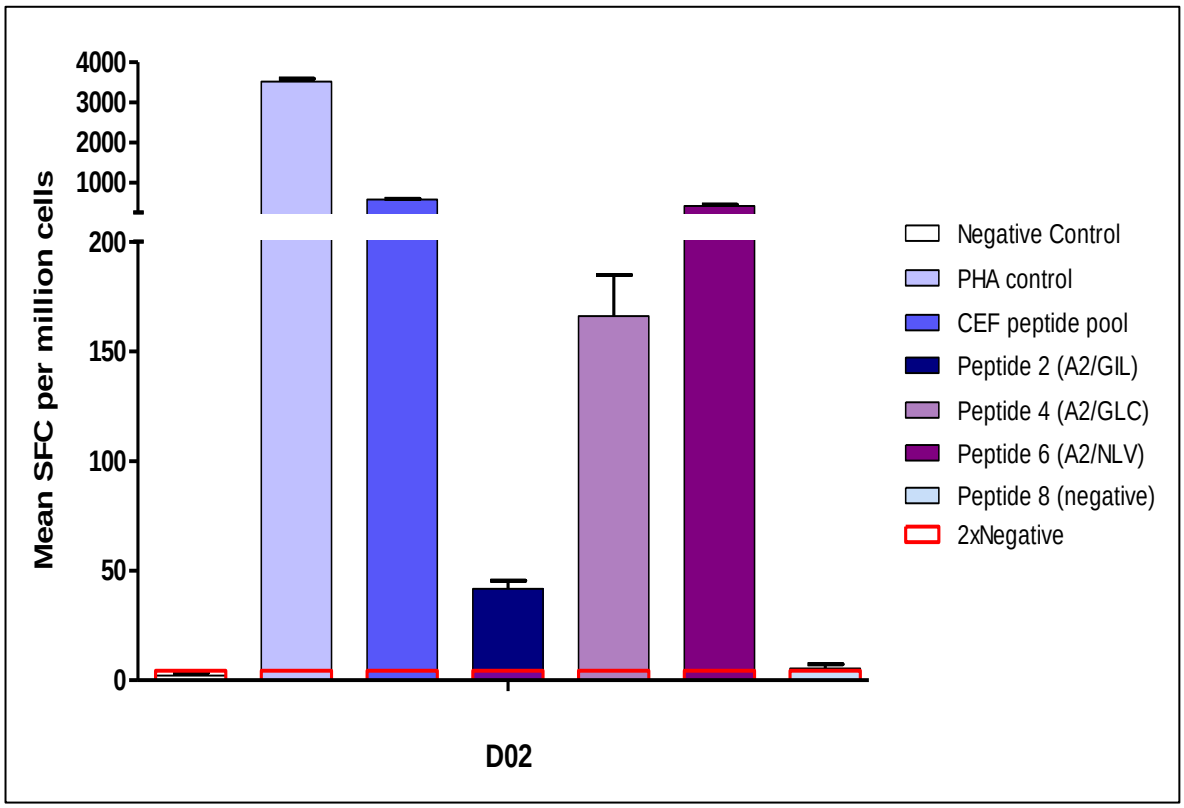

Figure 2: Number of SFC per Million Cells for Sample D02

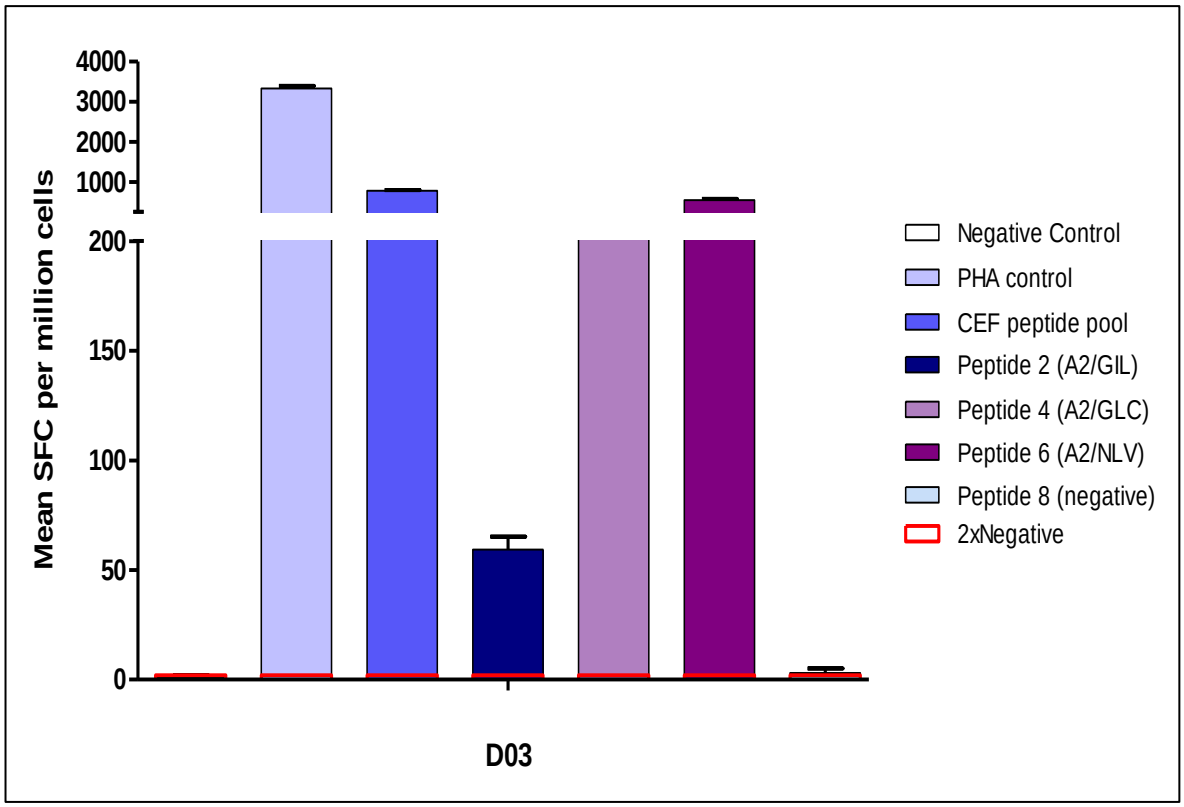

Figure 3: Number of SFC per Million Cells for Sample D03

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4.1.2 Tabulated Data

The mean results from all test wells are tabulated below. An un-paired two-tailed student's *t*-test was performed to analyze the samples for statistical significance. A result is deemed significant if the mean SFC per million cells minus two standard errors is greater than 2x the respective negative control wells, and where $p \leq 0.05$. Significant stimulation is highlighted in blue.

Table 4. Tabulated Data for All Donors

Donor ID	Stimulant	Mean SFC/1x10⁶	SEM	*p* Value
BD02_Setup 1	Media	0.9	0.9	
	PHA Control	2746.3	44.6	< 0.001
	CEF Pool Control	541.2	19.1	< 0.001
	Peptide 2 (A2/GIL)	44.9	5.2	< 0.001
	Peptide 4 (A2/GLC)	114.2	9.6	< 0.001
	Peptide 6 (A2/NLV)	346.4	19.2	< 0.001
	Peptide 8 (Negative)	3.7	1.2	0.092
BD02_Setup 2	Media	2.1	1.4	
	PHA Control	3520.9	74.7	< 0.001
	CEF Pool Control	579.8	16.0	< 0.001
	Peptide 2 (A2/GIL)	41.8	3.6	< 0.001
	Peptide 4 (A2/GLC)	166.1	18.7	< 0.001
	Peptide 6 (A2/NLV)	419.1	29.7	< 0.001
	Peptide 8 (Negative)	5.4	2.0	0.209
BD02_Setup 3	Media	1.0	1.0	
	PHA Control	3329.2	67.0	< 0.001
	CEF Pool Control	777.1	15.4	< 0.001
	Peptide 2 (A2/GIL)	59.3	5.9	< 0.001
	Peptide 4 (A2/GLC)	215.7	22.9	< 0.001
	Peptide 6 (A2/NLV)	547.0	28.1	< 0.001
	Peptide 8 (Negative)	3.1	2.1	0.401

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5 SUMMARY AND CONCLUSIONS

Responses to Positive and Negative Controls

Across all setups, responses to media alone (negative control) were less than 40 SFC per million and all BD02 samples achieved responses to PHA over 200 SFC per million, passing the assay acceptance criteria set for donors sourced, processed and cryopreserved by ProImmune. All PHA responses were significant.

All BD02 samples achieved a minimum response of 80 SFCs per million to CEF peptide pool across all three setups, and all responses were significant.

Responses to Test Articles

Significant responses to Peptides 2, 4 and 6 were obtained in all three independent setups. No significant responses were observed to Peptide 8 across all setups.

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